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Strawberry chlorotic fleck: Identification and characterization of a novel *Closterovirus* associated with the disease

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Abstract

Chlorotic fleck, a strawberry disease caused by a graft and aphid transmissible agent, was identified more than 45 years ago in Louisiana. Since its discovery there has been no additional information on the agent that causes the disease. The mode of transmission implies that a virus is the causal agent of chlorotic fleck. We identified four closteroviruses in the single chlorotic fleck infected strawberry clone known to exist in the United States. Sequence analysis indicated that two of the viruses are novel and one of them is closely related to members of the *Closterovirus* genus, the aphid-transmitted viruses in the family *Closteroviridae*, a feature that is in accordance with the aphid transmissibility of the chlorotic fleck agent. The genome of the novel *Closterovirus*, designated as Strawberry chlorotic fleck associated virus exceeds 17 kilobases and encodes 10 open reading frames, including the signature closterovirus genes as well as a gene without obvious homologs in the family. RNA folding predicted a pseudoknot structure near the 3′ terminus of the virus that may be involved in template recognition by the viral polymerase. Phylogenetic analysis indicates that Strawberry chlorotic fleck associated virus is most closely related to *Citrus tristeza virus* among sequenced members of the family. Detection protocols have been developed and the virus was detected in several strawberry plants from production fields.

Keywords: *Closteroviridae*; *Crinivirus*; Detection; Phylogenetic analysis

1. Introduction

During the last six years several strawberry producing areas in western North America, exhibit declining symptoms that have been attributed to viruses since the majority of the symptomatic plants were found infected with four or more viruses (Tzanetakis, 2004). Until recently only the minority of the more than 20 virus and virus-like diseases of strawberry (Martin and Tzanetakis, 2006) could be detected with laboratory-based techniques. This is one of the reasons that grafting is still routinely used for detection of the graft-transmissible agents that infect strawberry. The recent strawberry disease situation in western North America led to the investigation of the possibility that causal agent(s) of strawberry graft-transmissible diseases are associated with the decline symptoms and an effort is underway to develop laboratory tests for each of these agents. One of those diseases is chlorotic fleck (CF).

CF was first identified in Louisiana (Horn and Carver, 1962). The graft-transmissible agent can be transmitted by the cotton aphid (*Aphis gossypii*) and causes downward curling and distortion on both *Fragaria vesca* and *F. virginiana* indicators. The disease was named after the chlorotic lesions observed sometimes on *F. vesca* indicators (Fulton, 1987; Fig. 1). Plants of susceptible cultivars infected with the CF agent had a 40–70% runner reduction (Fulton, 1987). The epidemiology of the agent indicated that it may be a virus.

The National Clonal Germplasm Repository (NCGR) in Corvallis, Oregon, has the only known chlorotic fleck infected strawberry clone known to exist in the United States. The two pallidosis agents, Strawberry pallidosis associated virus (Tzanetakis et al., 2004) and *Beet pseudo-yellows virus* (Tzanetakis et al., 2003), and two novel viruses were found in the plant. Both novel viruses belong in the *Closteroviridae* but in different genera, the first in the genus *Crinivirus* and the second in the genus *Closterovirus*, the aphid-borne members in the family.

The *Closteroviridae* is one of the most diverse families of plant viruses. They have long filamentous virions of

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Fig. 1. Symptoms of chlorotic fleck disease on NCGR CFRA 9018. Arrowheads point to the chlorotic flecks on this *Fragaria vesca* clone.

700–2000 nm × 12 nm that contain the mono-, bi- or tripartite positive strand single-stranded RNA genomes that range in size from over 15 to almost 20 kilobases. The family is divided in three genera according to genome organization: *Ampelovirus*, *Closterovirus*, *Crinivirus*. *Ampelovirus* includes the mealybug-borne, *Closterovirus* the aphid-borne and *Crinivirus* the whitefly-borne members of the family. In addition there are several unassigned members demonstrating the diversity in the family. Several members of the family cause devastating diseases (Karasev, 2000) and given the losses suffered by the strawberry growers in the western coast of North America further steps were taken to characterize the novel closteroviruses, develop detection protocols and study the epidemiology and their potential role in strawberry decline.

This communication presents work performed towards the biological characterization of the novel *Closterovirus* as well as the development of a detection protocol. The complete nucleotide sequence of the virus designated as Strawberry chlorotic fleck associated virus (SCFaV) has been determined and genome and phylogenetic analyses were performed. Data on the presence of the virus in strawberry producing areas in the western North America is presented.

2. Materials and methods

2.1. Virus transmissions

Eleven herbaceous species (*Chenopodium quinoa*, *Cucumis sativus*, *Nicotiana occidentalis*, *N. benthamiana*, *Phaseolus vulgaris*, *Spinacia oleracea*, *Vigna sinensis*, *Brassica rapa*, *Hibiscus esculentus*, *Capsicum capsa* and *Gomphrena globosa*) were inoculated with chlorotic fleck infected tissue from NCGR accession CFRA 9018. The tissue was ground in 0.1× PBS pH 7.4 (1:20, w/v) in the presence of 2% nicotine (v/v). Carborundum (600 mesh) was dusted on the leaf surface of the indicators to facilitate delivery of the agent(s) into the indicator plants. Five to eight plants of each species were inoculated and tested for the presence of SCFaV approximately one month post-inoculation.

2.2. Virus and nucleic acid extractions

Double-stranded RNA (dsRNA) was extracted from NCGR accession CFRA 9018 as described previously (Tzanetakis and Martin, 2005). Total RNA for sequencing and reverse-transcription polymerase chain reaction (RT-PCR) detection was extracted as described for Blackberry yellow vein associated virus (BYVaV) (Susaimathu et al., 2006).

2.3. Cloning and sequence analysis

Complementary DNA was acquired from dsRNA and cloned as described (Tzanetakis et al., 2005a) without the use of restriction endonucleases. Recombinant plasmids were screened by PCR for large inserts and were sequenced at Macrogen Inc. (Seoul, South Korea) using an ABI3730 XL automatic DNA sequencer.

Data obtained were compared with sequences found in databases using blastn and blastx (Altschul et al., 1997). SCFaV-specific sequences were used in the development of oligonucleotide primers (Table 1) for RT-PCR amplification, performed as described previously (Tzanetakis et al., 2005b). After determining the ends using RACE (Tzanetakis et al., 2005b) specific primers were developed for the exact 5′ and 3′ termini and RT-PCR was performed to verify that the region was part of the virus genome. The obtained amplicons were cloned into TOPO pCR 4.0 vector (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. The sequences of the PCR amplicons, at least three clones of the amplicons, and clones obtained by shotgun cloning, where applicable, were used to create the consensus sequence utilizing CAP3 (Huang and Madan, 1999). The SCFaV sequence has been deposited in Genbank and given the accession number DQ860839.

Protein alignments and phylogenetic analysis was performed on ClustalW (Thompson et al., 1994). Comparison of the SCFaV protein sequences with orthologous genes of other members of the *Closterovirus* genus was done on GatMat (Campanella et al., 2003). The secondary structure of SFCaV RNA was predicted with Mfold (Zuker, 2003).

2.4. Detection

The techniques described for BYVaV detection (Susaimuthu et al., 2006) were also applied in SCFaV detection, with the exception that the RT template in the PCR reaction made 10% of the total volume of the reaction. Primers CPh detF and CPh detR (Table 1) that amplify a 392 bases fragment of the coat protein homolog (CPh) gene of the virus were used for routine detection of the virus. The PCR program was the same described for BYVaV. A total of 189 strawberry plants from California were tested for the presence of the virus.

Table 1
List of the oligonucleotide primers used in the amplification and detection of Strawberry chlorotic fleck associated virus

Primer name	Nucleotide sequence (5′–3′)
Genome amplification primers	
dT_2	GGCCACGCGTCGACTAGTAC$(T)_{18}$
AAP	GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG
Beg R	GCGAACAGGCAGCTTGGACTTA
1a 1030RC	AGTCAAAGCCACTTGCGATTGC
1a 1030F	GCAATCGCAAGTGGCTTTGACTG
1a 1700R	GTTAAGCAAAGCCATGAAGAGAGAGACTAAGCATT
1a 1700F	TTTGAATGCTTAGTCTCTCTCTTCATGGC
1a 2700R	CTAAAGCATATGTTTTTCCCCCACC
1a 2700F	GGTGGGGGAAAAACATATGCTTTAGT
Mid R	GTCTAAGTCAGATCTCTCGTAACAGTTCAT
Mid F	CGGTCTCGTCATCGCTGTGGTAGT
Hsp mid R	ATCAAAAGGAATCGGTGCTC
Hsp mid F	GCTTGACAAGCGCCAGTAACG
CPh end R	ACGACGCCTTCTGTAGTGAATCGAAA
CPh end F	CCGCAAAAGAACGTACGATACGCA
p20 R	TTGCTTGAGTCCATCGGTACGCCGTT
p20 F	CAACAATCACTACGTTTCATGTATTCAC
Detection primers	
SCFaV CPh detF	CGTGGGTGATCGCTAC
SCFaV CPh detR 392 bp	ATACGACGCCTTCTGT

3. Results

NCGR accession CFRA 9018 was found infected with four viruses. All viruses belong to the *Closteroviridae*, and two are novel viruses. Sequence analysis indicated that three of the viruses, *Beet pseudo-yellows virus*, Strawberry pallidosis associated virus and one of the novel viruses belong to the *Crinivirus* genus. The fourth, SCFaV was most closely related to the genus *Closterovirus* and therefore a potential causal agent of CF.

The complete nucleotide sequence of SCFaV was determined. It consists of 17,039 nucleotides (nt) and encodes ten open reading frames (ORF) (Fig. 2). The 5′ untranslated region is 227 nt long. Secondary structure analysis showed that the region is not highly structured, a feature shared among the members of the genus (data not shown).

The first ORF (1a) codes for a multifunctional 312 kDa protein. Sequence analysis revealed the presence of two papain-like proteases, a methyltransferase and a helicase domain. The proteases, found at the N-terminus of the polyprotein (aa 1–439 and 440–650), have molecular weight of 42 and 24 kDa, respectively, and share 25% aa identities (44% similarities) in their C-terminus (Fig. 3). After cleavage of the two proteases, the remaining 240 kDa protein encodes the methyltransferase domain in its N-terminus. The conserved methyltransferase motifs have been identified between residues 736 and 1064 (Rozanov et al., 1992). The region has more than 60% aa similarities with all sequenced members of the genus sequenced to date. Similar to other closteroviruses, a region of over 1300 aa separate the methyltransferase and helicase domains. No enzymatic motifs were identified in the region and the similarity with homologous regions of other members of the genus was about 40%. The helicase domain found at the C-terminus of the polyprotein (aa 2401–2674; Marchler-Bauer et al., 2003), shows the same degree of similarity to orthologous domains

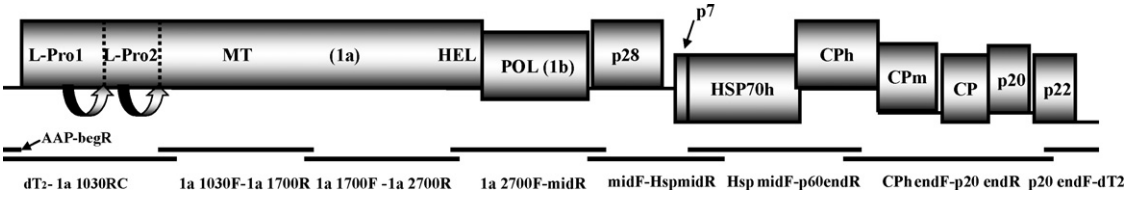

Fig. 2. Genomic organization of Strawberry chlorotic fleck associated virus. Abbreviations: L-Pro1 and L-Pro2, leader papain-like protease 1 and 2, respectively; MT, methyltransferase; Hel, helicase; Pol, RNA-dependent RNA polymerase; HSP70h, heat shock protein 70 homolog; CPh, coat protein homolog; CPm, minor coat protein; CP, major coat protein. Bars represent the reverse transcription-polymerase chain reaction products used to acquire the genome of the virus. Oligonucleotide primers are listed in Table 1. Open reading frames are not to scale.

Fig. 3. Alignment of the C-termini of Strawberry chlorotic fleck associated virus leader proteases using ClustalW. Identical residues are found in black boxes, similar in grey. The arrowheads indicate the catalytic cysteine and histidine residues of the enzymes.

of other members of the genus with the methyltransferase domain.

The polymerase (1b) of the virus is probably expressed through a + 1 ribosomal frameshift as is presumed for other closteroviruses (Dolja et al., 2006). Primary sequence and RNA secondary structure analysis did not reveal any structures that would facilitate ribosome slippage but the frameshift may be induced by an antisense mechanism (Henderson et al., 2006). After the putative frameshift, the fusion 1a/1b would have MW of 366 kDa. The polymerase domain of the fusion protein encodes all eight conserved motifs identified by Koonin (1991) between aa 2896–3134 and has more than 66% aa identity and 80% aa similarity to all members of the genus for which the polymerase gene has been sequenced.

Downstream of the polymerase, an ORF codes for a 28 kDa putative protein that does not show significant similarity with any protein in the databases. An interesting feature is the presence of a transmembrane domain at the C-terminus of the protein. The predicted domain spans from aa 220–242 leaving the majority of the peptide in the cytosol and six residues in the exterior. The 23 aa transmembrane region shows 35% aa identity (61% aa similarity) to *Citrus tristeza virus* (CTV) p33 transmembrane domain and 23% aa identity (45% similarity) with *Beet yellow stunt virus* (BYSV) p30 transmembrane domain.

The five proteins involved in closterovirus movement (Dolja et al., 2006) are found downstream from the p28 ORF. A 64 aa hydrophobic protein has a cysteine residue at position 3, probably used in the dimerization of the protein (Peremyslov et al., 2004) and a transmembrane domain between residues 12 and 34, features found in all small hydrophobic proteins encoded by members of the *Closterovirus* genus. The transmembrane region of the small hydrophobic proteins of SCFaV and CTV show 32% aa identity and 64% similarity, numbers similar to the transmembrane domains of SCFaV p28 and CTV p33. The heat shock protein 70 homolog (Hsp70h), the hallmark protein of all closteroviruses is 592 aa long with MW of 65 kDa and encodes the five Hsp conserved motifs (Bork et al., 1992). The protein shows about 40% aa identity and 60% aa similarity with the orthologous proteins of other closteroviruses. The coat protein homolog (CPh) of SCFaV is 532 aa long with an estimated MW of 61 kDa. The conserved Arg and Asp residues of filamentous virus coat proteins (Dolja et al., 1991) are found at position 416 and 455, respectively. The homology with other *Closterovirus* CPh ranges from about 30 to 35% aa identity and 50% aa similarity. The two coat proteins of the virus are found downstream from the CPh. The minor coat protein (CPm) is 20 aa longer that the major coat protein (CP) having the 'CPm box' at the N-terminus (Alzhanova et al., 2001) and has MW of 25 kDa. The major coat protein is 195 aa long with MW of 21 kDa. The conserved Ser, Arg and Asp residues of the coat proteins are found at positions 88, 138, 179 of the CPm and 65, 112, 149 of the CP, respectively. A putative protein of 20 kDa was found downstream of the genes encoding the structural of SCFaV. The protein did not have significant similarity with any protein in the databases as is the case with all other *Closterovirus* proteins found in the same position of the genome. The final ORF of the genome encodes for a 22 kDa protein that shows similarity with gene silencing suppressors of other closteroviruses (Reed et al., 2003) including the conserved motif near the C-terminus of the protein (Tzanetakis et al., 2005b).

The 3′ untranslated region of SCFaV is 214 nt long. The predicted secondary structure of the virus resembles that of *Potato yellow vein virus* (Livieratos et al., 2004), folding in several stem-loops and a putative pseudoknot near the 3′ terminus (Fig. 4).

Phylogenetic analysis using the conserved motifs of the polymerase and the Hsp70h of all sequenced closteroviruses (Fig. 5) revealed that SCFaV is related most closely to CTV.

Mechanical inoculations onto herbaceous hosts failed to reveal an alternative host for the virus. Five out of the 189 plants from California, from areas with high incidence of strawberry viruses (Martin and Tzanetakis, 2006), tested positive for the virus.

4. Discussion

Closteroviruses are an emerging group of viruses, with several new members discovered recently (Martin et al., 2004; Segundo et al., 2004; Alkowni et al., 2004). While several members cause devastating diseases, e.g. CTV, Grapevine leafroll associated viruses, the group is grossly understudied in comparison to other virus groups. This is primarily due to the difficulty to transmit closteroviruses to herbaceous hosts, the low titer they

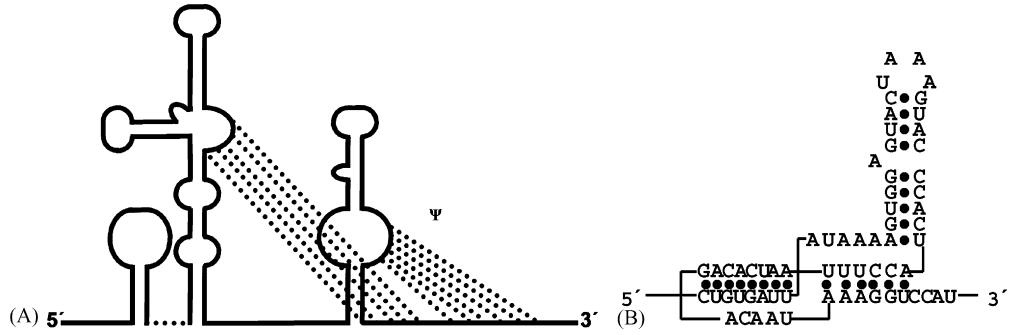

Fig. 4. (A) Predicted secondary structure of the 3′ terminus of Strawberry chlorotic fleck associated virus as predicted by Mfold. Dashed lines indicate the possible interactions between unpaired bases. Ψ indicates is the region of the putative pseudoknot. (B) The putative pseudoknot structure at the 3′ terminus of Strawberry chlorotic fleck associated virus.

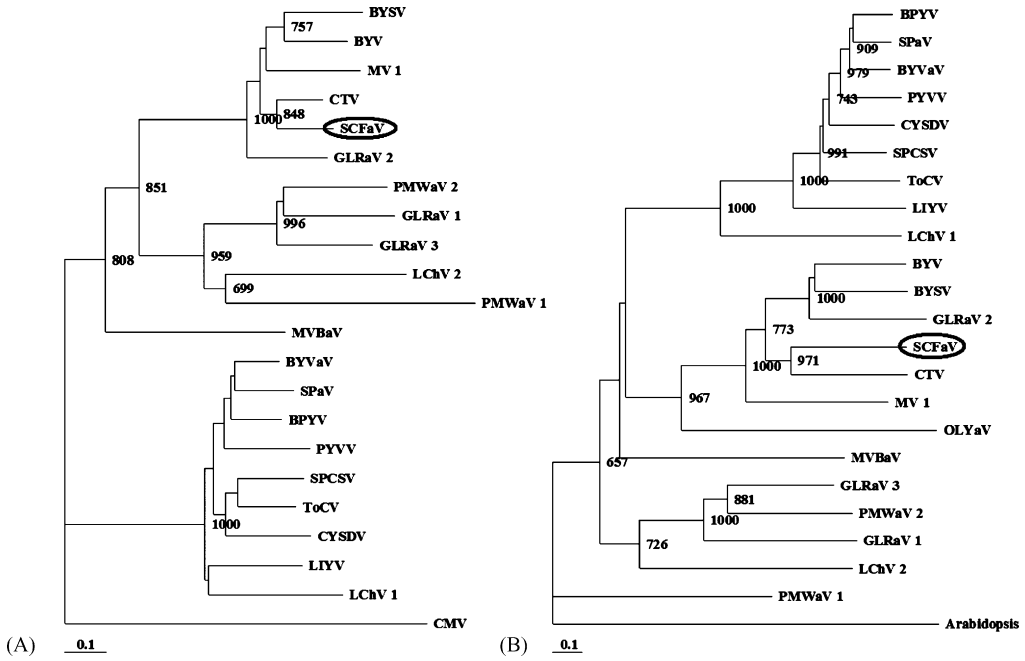

Fig. 5. (A) Phylogram of the polymerase conserved motifs of Strawberry chlorotic fleck associated virus and other closteroviruses. Abbreviations and GenBank accession numbers: BPYV, *Beet pseudo yellows virus*, NP940796; BYV, *Beet yellows virus*, NP733949; BYSV, *Beet yellow stunt virus*, AAC55659; BYVaV, Blackberry yellow vein associated virus, AAV40963; CTV, *Citrus tristeza virus*, NP733947; CMV, *Cucumber mosaic virus*, NP049324; CYSDV, *Cucurbit yellow stunting disorder virus*, AAM73639; GLRaV 1, *Grapevine leafroll associated virus 1*, AAF22738; GLRaV 2, *Grapevine leafroll associated virus 2*, AAC40856; GLRaV 3, *Grapevine leafroll associated virus 3*, AAC40705; LIYV, *Lettuce infectious yellows virus*, AAA61798; LChV 1, *Little cherry virus 1*, NP733945; LChV 2, *Little cherry virus 2*, AAP87784; MVBaV, Mint vein banding associated virus, AAS57939; MV 1, Mint virus-1, AAW32893; PMWaV 1, *Pineapple mealybug wilt associated virus 1,* AAL66709; PMWaV 2, *Pineapple mealybug wilt associated virus 2,* AAG13939; PYVV, *Potato yellow vein virus*, CAD89680; SCFaV, Strawberry chlorotic fleck associated virus, DQ860839; SPaV, Strawberry pallidosis associated virus, AY488137; SPCSV, *Sweet potato chlorotic stunt virus*, NP733939; ToCV, *Tomato chlorosis virus*, AAY21795. CMV is used as the outgroup. (B) Phylogram of heat shock protein 70 homolog of Strawberry chlorotic fleck associated virus and other closteroviruses. GenBank accession numbers: Arabidopsis, *Arabidopsis thaliana* putative heat shock protein 70, AAN71949; BPYV, AAQ97386; BYSV, AAC55662; BYV, NP041872; BYVaV, AAV40966; CTV, NP042864; CYSDV, NP851572; GLRaV 1, AAF22740; GLRaV 2, AAR21242; GLRaV 3, NP813799; LIYV, NP619695; LChV 1, NP045004; LChV 2, AF531505; MVBaV, AAS57941; MV 1, AAW32895; PMWaV 1, AAL66711; PMWaV 2, AAG13941; OLYaV, *Olive leaf yellowing associated virus*, AJ440010; PYVV, CAD89682; SCFaV, DQ860839; SPaV, AAO92347; SPCSV, NP689401; ToCV, AF024630. The Arabidopsis protein is used as the outgroup. Bootstrap values are shown as percentage value and only the nodes over 60% are labeled. The bars represent 0.1 amino acid changes per site.

reach in their hosts and the difficulty to purify due to the fragility of the flexuous virions.

No closterovirus was known to infect strawberry and rosaceous hosts until recently. The NCGR accession CFRA 9018 revealed the presence on two new closteroviruses in strawberry in addition to the two identified previously.

The complete nucleotide sequence of SCFaV has been determined. The 5′ proximal region of the genome has an organization similar to CTV while the 3′ region is organized similar to BYSV, a virus partially sequenced at the time of submission of this manuscript. All proteins of SCFaV are more closely related to the CTV orthologs than to any of the other closteroviruses, and phylogenetic analysis verified the observation (Fig. 5). In the evolution of the *Closterovirus* genus, SCFaV is an intermediate species between *Beet yellows virus* and Mint virus-1, the simplest closteroviruses, and the most complex, CTV. It is only the third *Closterovirus* that encodes two proteases after CTV and *Grapevine leafroll associated virus-2* (Peng et al., 2001; Meng et al., 2005), but does not have an ortholog for CTV p23, an RNA silencing suppressor (Lu et al., 2004). The similarity of the transmembrane domains of p28 and p7 point to the close relationship of the SCFaV and CTV. While CTV p33, BYSV p30 and SCFaV p28 do not show significant homology, the conservation of the transmembrane domain may be indicative of the active role that the region has in the function of these proteins. Since p33 may be a host determinant (Satyanarayana et al., 2004), the roles of these proteins may have diverged to facilitate different functions in the hosts the viruses infect.

The pseudoknot at the 3′ terminus may be involved in template recognition by the polymerase as is the case with structural features at the 3′ termini of other viruses (Huang et al., 2001; Osman et al., 2000).

We have tried several times to isolate the pallidosis agents from strawberry without success. The presence of four closteroviruses in accession CFRA 9018 did not facilitate the purification of SCFaV virions, and thus, we tried to transmit the virus to a herbaceous host with the ultimate goal to purify the virus and develop antibodies for an immunological test for SCFaV. While members of the group can be transmitted to herbaceous hosts with some difficulty, we were unable to transmit SCFaV into any of the 11 species tested. The RT-PCR test developed for the virus was used successfully in field detection of SCFaV in

California, where five of the almost 200 plants tested were positive for SCFaV. These findings indicate that the virus is present in strawberry fields and may play a role in strawberry decline.

While there were plants that tested positive for SCFaV, the field material arrived to our lab as single leaflets. Thus, it was not possible to establish trials for transmission of the virus using the *Aphis gossypii* or other species of aphids. Aphid transmission trials using the CFRA 9018 plant have been unsuccessful (data not shown). This isolate has been propagated vegetatively in strawberry, possibly without any aphid transmissions for more than 40 years which may have resulted in the loss of aphid transmissibility due to accumulation of mutations (Atreya et al., 1991).

The next steps to be taken include identification of an infected field plant and testing of several aphid species for their ability to transmit the SCFaV onto indicators. Possible transmission and development of the chlorotic fleck symptoms will verify that SCFaV is the causal agent of the disease.

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